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pH-Sensitive metal-free carbon monoxide prodrugs with tunable and predictable release rates†

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Carbon monoxide prodrugs with triggered release profiles are highly desirable for targeted CO delivery to minimize their untoward side-effects. Herein, we describe a series of pH-sensitive metal-free CO prodrugs which are stable under acidic conditions and yet begin to release CO in response to increases in pH with tunable and predictable release rates.

Carbon monoxide, generated by heme oxygenase-mediated heme degradation, is an endogenous signaling molecule with strong cytoprotective and anti-inflammatory effects, among others.[1] For example, there have been remarkable efforts and success in studying CO for the treatment of experimental ulcerative colitis (UC).[2] Because of the significant therapeutic potential of CO, much effort has been devoted to the search for CO-releasing molecules (CO-RMs) with either spontaneous release upon dissolution[3] or controllable release in response to various stimuli, including photo-,[4] enzyme-,[5] and oxidation-sensitive[6] and encapsulated CO-RMs.[7] Previously, we have successfully developed a series of metal-free CO prodrugs by using inter- or intra-molecular Diels–Alder (DA) reactions to trigger CO release (Fig. 1).[2b,8] Among all the successes, pH-sensitive CO release is considered to be a very important method for achieving local delivery of CO. For example, CORM-A1 was reported to be pH-sensitive,[9] which releases CO under acidic pH and is thus ideal for gastric delivery. Herein, we describe a series of pH-sensitive CO prodrugs, which are metal-free and stable under acidic conditions. They begin to release CO at pH above 5 with a tunable and predictable release rate for future applications. Such prodrugs also complement CORM-A1 in allowing the prodrug to survive the stomach for CO delivery to the lower gastric intestinal (GI) system.

The design takes advantage of the fact that intermediate **I** under the right conditions can undergo a facile cheletropic reaction to release CO as previously demonstrated (Fig. 1). Thus, we were interested in the design of precursors of this intermediate.

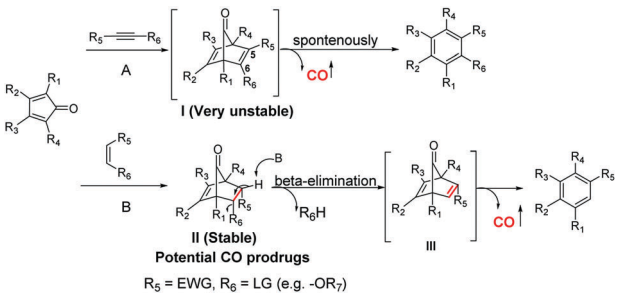

Fig. 1 Schematic illustration of the beta-elimination triggered CO release (EWG: electron withdrawing group; LG: leaving group).

Very interestingly, the cycloaddition product **II** between an alkene and cyclopentadienone is thermally stable (Fig. 1), and CO release from **II** can only be initiated when the temperature is over 180 °C.[10] Therefore, compound **II** could serve as a potential CO prodrug, provided that a double bond between C5 and C6 can be easily formed under physiological conditions to yield intermediate III for spontaneous CO release. In doing so, the key is to regenerate a double bond between the C5 and C6 positions under physiological conditions. In order to devise pH-sensitive CO prodrugs for delivery to the lower GI, we employed base catalyzed beta-elimination as a strategy to form the double bond between C5 and C6 under physiological conditions (Fig. 1, R_5 is chosen for positioning an electron-withdrawing group, and R_6 is a leaving group). CO prodrugs as such are expected to be very stable under acidic conditions, and would only undergo beta-elimination to release CO under basic or possibly neutral conditions. In addition, by changing different R_6 groups with various leaving-group abilities, we are able to tune the CO release rate, which is a very important parameter in gasotransmitter prodrugs.[2b,8b,11]

To establish the proof of concept, we designed and synthesized several potential pH-sensitive CO prodrugs **BW-CO-201–205** (Scheme 1). An aldehyde group is chosen as R_5 due to its strong electron withdrawing ability. Despite being very good leaving groups, we sought to avoid halides to minimize their alkylation capacity and issues related to the formation of hydrogen

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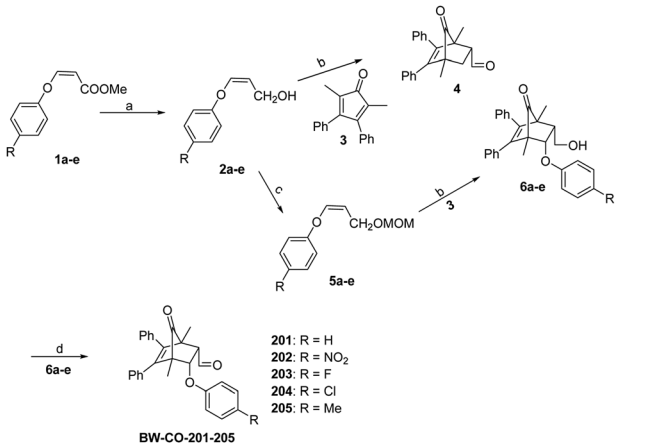
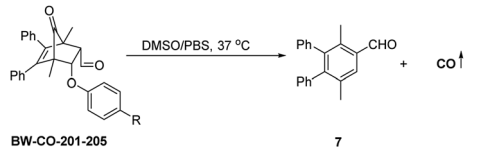


Scheme 1 The chemical synthesis of the metal-free CO prodrugs. *Reagents and conditions*: (a) DIBAL, CH_2Cl_2, −78 °C, 1–2 h, 75–80%; (b) xylene, 170 °C, 12 h; then HCl, MeOH, reflux, 1 h, 50–60%; (c) DIPEA, MOMCl, CH_2Cl_2, r.t., 3 h, 85–90%; and (d) CH_2Cl_2, PCC, reflux, 30 min–1 h, 60–73%.

halides, which are strong acids.[12] As a result, substituted phenols were chosen to achieve a balance between the leaving ability and reactivity. As shown in Scheme 1, CO prodrugs **BW-CO-201–205** were readily synthesized in 4 steps. Specifically, compounds **1a–e**, which were obtained following a literature procedure,[13] were reduced using DIBAL to yield the corresponding alcohols **2a–e**. The DA reaction between **2a** (R = H) and **3** failed to afford the desired product **6a**, and compound **4** was obtained instead. The formation of compound **4** is presumably due to the generation of acrylaldehyde *in situ* from compound **2a** *via* double bond migration, tautomerization, and elimination of the phenol group. Therefore, the hydroxyl group in compounds **2a–e** was protected with MOMCl to form **5a–e** before the DA reaction. As expected, compounds **6a–e** were successfully obtained primarily as endo products (Fig. S1 and S2, ESI†) after the DA reaction and deprotection of the MOM group. The final CO prodrugs **BW-CO-201–205** were obtained upon oxidation of the hydroxyl group in compounds **6a–e** using PCC in CH_2Cl_2 at reflux temperature. No beta-elimination was observed in the process, indicating the desirable stability of CO prodrugs **BW-CO-201–205** in an organic solvent. We also tested the stability of **BW-CO-201** in organic solvents (*e.g.* $CDCl_3$) at 37 °C, and no beta-elimination products were observed even after one week of incubation (Fig. S3, ESI†).

With these compounds in hand, we set out to test whether they would undergo a facile beta-elimination reaction to release CO under near physiological conditions. To our delight, all compounds readily underwent beta-elimination in a mixed aqueous solution (Table 1) with concomitant release of CO. The CO release was confirmed by a thorough elucidation of the structure of by-product **7** and a widely accepted CO-myoglobin assay (Fig. S4, ESI†). As shown in Table 1, by varying the leaving groups, the CO release half-life can be readily tuned from 20 min (**BW-CO-202**) to 1.2 h (**BW-CO-205**). Generally, the more electron withdrawing the R group is, the faster the CO release is. The observed CO release rate constant (Table 1) also showed a very good correlation with the Hammett constant of the R groups (Fig. 2, $R^2 = 0.99$), which is

Table 1 The CO release kinetics of **BW-CO-201–205**



CO prodrugs	R	Hammett constant[a]	k^b (h^{-1})	$t_{1/2}{}^c$ (h)
201	H	0	0.9	0.77
202	NO_2	0.78	2.09[d]	0.33
203	F	0.06	1.07	0.65
204	Cl	0.23	1.2	0.58
205	Me	−0.17	0.58	1.2

[a] The Hammett constant of the R group at the *para*-position was extracted from a reported literature.[14] [b] CO release rate constant was determined in 30% of DMSO in PBS (pH = 7.4) at 37 °C by using HPLC. [c] CO release half-life was calculated according to $t_{1/2} = 0.693/k$. [d] The CO release kinetics of **BW-CO-202** was determined by monitoring the formation of 4-nitrophenol using UV absorbance at 400 nm.

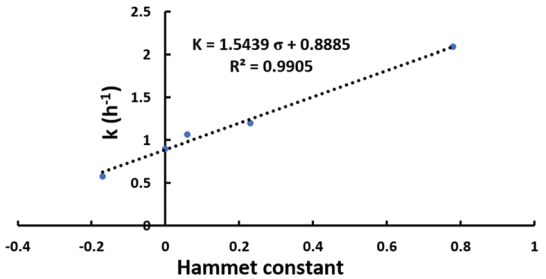


Fig. 2 The correlation of CO release rate (k) to the Hammett constant (σ).

valuable for quantitatively predicting the half-lives/release rates of newly synthesized CO prodrugs.

Having confirmed the CO release from **BW-CO-201–205**, we next tested whether CO release is sensitive to the pH value of the buffer used. Toward this end, **BW-CO-203** was employed to study the CO release profiles in a buffer solution at different pH values by monitoring the consumption of the prodrug using HPLC. As expected, the CO release from **BW-CO-203** is dependent on the pH of the buffer solution, and is very sluggish in an acidic buffer solution (pH = 3) with a half-life of over 9 h. However, when the pH value reached 5, the CO release rate is significantly increased with a half-life of 1.7 h. At pH 7.4 in PBS, the half-life decreased to 0.65 h. We also tested CO release from **BW-CO-203** in a simulated gastric fluid (SGF) without pepsin (pH = ~1), and, as expected, around 80% of the prodrug remained intact after 8 h of incubation at 37 °C (Fig. 3). Altogether, such CO prodrugs should be stable in the stomach for delivery to the lower GI.

Having confirmed CO release from **BW-CO-201–205**, we next tested whether those CO prodrugs could be used to deliver enough CO intracellularly to recapitulate CO-associated anti-inflammatory effects. All CO prodrugs were initially screened for their cytotoxicity against Raw 264.7 cells, and the results showed that no cytotoxicity was observed for these CO prodrugs along with their respective inactive products (**7** and substituted phenols) at concentrations up to 50 μM after 24 h of incubation, but all CO prodrugs showed obvious cytotoxicity when the

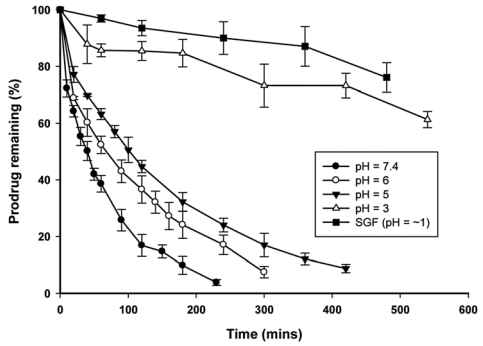


Fig. 3 CO release profiles of **BW-CO-203** in a buffer containing 30% of DMSO at 37 °C and different pH values. SGF: simulated gastric fluid without pepsin containing 0.2% of NaCl (w/v) and 0.7% of HCl (v/v).

concentration reached 100 μM (Fig. S15 and S16, ESI†). **BW-CO-203** and **205** were then chosen for determining CO's anti-inflammatory effects using an Elisa assay for TNF-α. Specifically, Raw 264.7 cells were pretreated with **BW-CO-203** and **205** and their respective inactive products for 4 h, followed by the treatment of LPS (1 μg mL^{-1}) for another 1 h. Then the cell supernatants were collected to determine the TNF-α level by using a commercially available Elisa kit. As shown in Fig. 4, both **BW-CO-203** and **205** dose-dependently inhibited LPS-induced secretion of TNF-α, and no similar effects were observed for their respective inactive products, suggesting that the observed TNF-α suppression effects were attributed to the CO released from **BW-CO-203** and **205**.

In order to further confirm intracellular CO release from these CO prodrugs, **BW-CO-203** and **205** were chosen for cell imaging studies using a reported CO fluorescent probe, COP-1.[15]

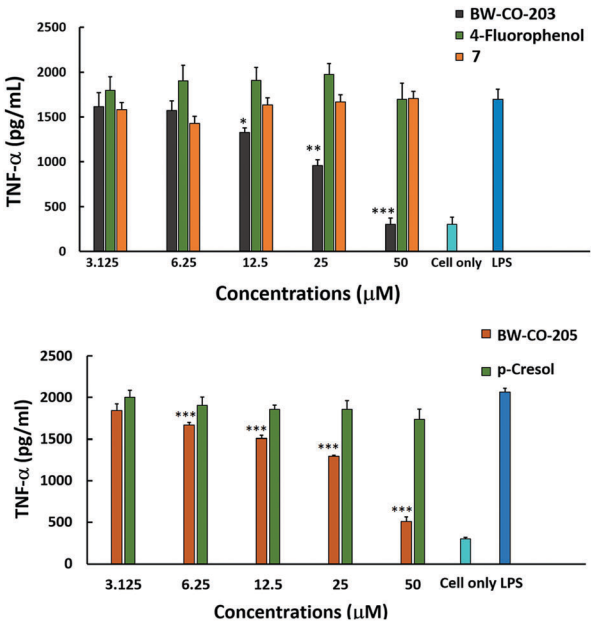


Fig. 4 The anti-inflammatory effects of **BW-CO-203/205** in Raw 264.7 cells. The mean of each concentration of **BW-CO-203** and **205** treated group was compared with the LPS-only group *via* a two-sample *t*-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

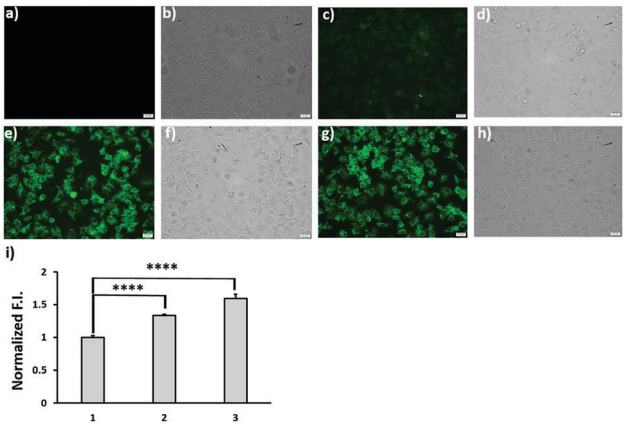


Fig. 5 The intracellular CO release from **BW-CO-203/205**. (a and b) The FITC channel and bright field of cell only; (c and d) the FITC channel and bright field of COP-1 (1 μM); (e and f) the FITC channel and bright field of COP-1 (1 μM) + **BW-CO-203** (50 μM); (g and h) the FITC channel and bright field of COP-1 (1 μM) + **BW-CO-205** (50 μM); (i) the fluorescence quantification (λ_{ex}: 485 nm, λ_{em}: 525 nm) of cells treated with COP-1 (1), **BW-CO-203** + COP-1 (2) or **BW-CO-205** + COP-1 (3). The experiment was repeated six times. ****: $p < 0.0001$. Scale bar: 20 μm. The images were taken using a 40× objective.

As shown in Fig. 5, the cells co-treated with COP-1 and **BW-CO-203** or **205** showed much enhanced green fluorescence compared to the ones treated with COP-1 only, indicating ready intracellular CO release from **BW-CO-203** and **205**.

In conclusion, CO prodrugs with a triggered release mechanism are highly desirable for local delivery of CO to minimize unintended side effects. We have successfully developed a series of pH-sensitive metal-free CO prodrugs with a tunable and predictable release rate, which are very stable under acidic conditions (*e.g.* simulated gastric fluid), and yet elicit CO release in a buffer in a pH-sensitive fashion. Two CO prodrugs (**BW-CO-203/205**) were studied as examples for their ability to deliver enough CO intracellularly to recapitulate CO-associated anti-inflammatory effects. Beyond the chemistry work described, we are in the process of examining these CO prodrugs in animal model studies.

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